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The combined effects of X-ray radiation and hindlimb suspension on bone loss

Dan XU[1,†], Xin ZHAO[2,†], Yi LI[2], Yinli JI[3], Jiangyan ZHANG[4], Jufang WANG[1], Xiaodong XIE[2,*] and Guangming ZHOU[1,*]

[1]Department of Space Radiobiology, Key Laboratory of Heavy Ion Radiation Biology and Medicine, Institute of Modern Physics, Chinese Academy of Sciences, 509 Nanchang Road, Lanzhou, China
[2]Key Laboratory of Preclinical Study for New Drugs of Gansu Province, School of Basic Medical Sciences, Lanzhou University, 222 Tianshui Road, Lanzhou 730000, China
[3]Gansu Hualing Biotechnology Research Center, 32 Central West Road, Lanzhou 730000, China
[4]Institution of Pathology, School of Basic Medical Sciences, Lanzhou University, 222 Tianshui Road, Lanzhou 730000, China
*Corresponding authors. Department of Space Radiobiology, Key Laboratory of Heavy Ion Radiation Biology and Medicine, Institute of Modern Physics, Chinese Academy of Sciences, 509 Nanchang Road, Lanzhou, China. Tel: +86-931-496-9164; Fax: +86-931-496-9164; Email: zhougm@impcas.ac.cn. Key Laboratory of Preclinical Study for New Drugs of Gansu Province, School of Basic Medical Sciences, Lanzhou University, 222 Tianshui Road, Lanzhou 730000, China; Tel: +86 13150022776. xdxie@lzu.edu.cn
†Contributes equally to this work.

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Outer space is a complex environment with various phenomena that negatively affect bone metabolism, including microgravity and highly energized ionizing radiation. In the present study, we used four groups of male Wistar rats treated with or without four-week hindlimb suspension after 4 Gy of X-rays to test whether there is a combined effect for hindlimb suspension and X-ray radiation. We tested trabecular parameters and some cytokines of the bone as leading indicators of bone metabolism. The results showed that hindlimb suspension and X-ray radiation could cause a significant increase in bone loss. Hindlimb suspension caused a 56.6% bone loss ($P = 0.036$), while X-ray radiation caused a 30.7% ($P = 0.041$) bone loss when compared with the control group. The combined factors of hindlimb suspension and X-rays exerted a combined effect on bone mass, with a reduction of 64.8% ($P = 0.003$).

Keywords: bone loss; hindlimb suspension; X-ray radiation; trabecular parameters

INTRODUCTION

Astronauts on missions beyond Earth's orbit will be required to live and work in space for long periods of time [1]. The varied and challenging environment of space may lead to a series of diseases such as orthostatic intolerance, immune deregulation, muscle atrophy and psychological disease [2]. Orthostatic intolerance, which is caused by bone loss, may become the major side effect of spaceflight, and no effective treatment exists [3]. In addition, missions in Low Earth Orbit result in decreased bone mineral density (BMD) of cortical and cancellous tissue and ~2.5% per month decrease in femoral head stance strength, as assessed by modeling [4].

In the complex environment of space, microgravity and ionization radiation may be the key factors in bone loss [5]. Since Morey-Holton and Globus summarized a method that used hindlimb suspension (HLS) to simulate microgravity effects in 2002 [6], HLS has become a widely accepted rodent model able to simulate the mechanical unloading experienced in microgravity. In one of a number of research investigations, rats underwent HLS for 2 weeks (followed by

Table 1. Study design combining radiation and HLS

	Non-radiation	X-radiation
Normally loaded	Control ($n = 10$)	X-rays ($n = 10$)
HLS	HLS ($n = 10$)	X-rays + HLS ($n = 10$)

a recovery period) in order to investigate hindlimb motion under microgravity [7].

Radiation can cause cell death through the abnormal repair of DNA damage [9]; low-dose radiation can result in non-lethal DNA damage [10]. Several studies have investigated the effects of high-dose radiation on bone cells *in vitro* and *in vivo*. For example, X-ray radiation within the range of 2.5–8 Gy has been found to inhibit the proliferation and activity of osteoblasts and osteoclasts [11–12].

During extended missions, astronauts are exposed to both solar and cosmic radiation; the high-energy protons and other charged particles can damage both shielding materials and biological systems. For Mars missions, cosmic rays (containing heavy ions and protons) and solar particle events (SPEs, composed mainly of protons) would be expected to occur randomly and could deliver a relatively high dose (up to 2 Gy) over a short period of time [13–15]. According to other research, a dose of ~2 Gy X-ray radiation is also a realistic possibility [16].

Although the bone loss associated with microgravity has been well characterized, the effects of radiation on the skeletal system are not yet well understood. Moreover, few publications have described the effects of both radiation and microgravity on bone loss. In this study, we used X-rays followed by unloading using HLS to investigate the combined effects of these space-simulating factors.

MATERIALS AND METHODS

Animals

Eight-week-old male Wistar rats were purchased from Gansu University of Traditional Chinese Medicine, China, and acclimatized for one week under standard vivarium conditions. The animals were fed for five weeks and then killed. The animals were grouped randomly (four groups, $n = 10$/group; Table 1) and all the rats were supplied with standard vivarium conditions. All animal procedures used were approved by the Institutional Animal Care and Use Committee at Lanzhou University.

Irradiation

Two groups of animals were irradiated (X-rays and X-rays + HLS) and the remaining experimental animals (control group and HLS) were subjected to a sham radiation procedure. The radiation for the rats was delivered one rat at a time. Whole-body radiation was administered once as 100 KVp X-rays generated by a Faxitron RX-650 (Faxitron Bioptics, Lincolnshire, IL, USA) housed at the Institute of Modern Physics, Chinese Academy of Sciences. A total dose of 4 Gy of X-rays was given over 275 s at a dose rate of 0.87 Gy/min, while the sham radiation procedure was performed in the same machine with a dose of 0 Gy of X-rays over 275 s. The dosimetry was as described previously [17–18]. After irradiation, the rats were immediately subjected to hindlimb unloading.

Hindlimb unloading

After the irradiation or sham procedure, two groups of rats were subjected to HLS (HLS and X-rays + HLS groups). HLS was performed with the Morey-Holton method with a minor modification [6]. Briefly, the rats were suspended at an angle of ~30° using a paper clip, adhesive tape, and a custom-built metal tail harness at the end of the paper clip. The tail harness was attached via a metal chain to a swivel buckle mounted on a guide wire running the length of the cage. Using this set-up, the rats were able to access all areas of the cage. The sham HLS group was set as the control group (with no hind limb suspension) and the rats could freely get the food and water. The whole process was maintained for four weeks.

Real-time PCR

After the rats were killed, the femurs were stored in liquid nitrogen at first and then stored at −80°C. Total RNA was extracted with TRIzol Reagent (Invitrogen, Shanghai, China). The RNA (1 μg) was reverse transcribed using M-MLV reverse transcriptase (Invitrogen), and 1 μg of cDNA was used for the PCR reactions. Real-time PCR was performed with Platinum SYBR Green qPCR Super Mix-UDG (Invitrogen) with the cycling conditions: 50°C for 2 min; 95°C for 5 min as an initial denaturing step; 45 cycles of 95°C for 20 s, 61.5°C for 30 s, and 72°C for 30 s. The melting curve was measured from 65–95°C, with 6 s holds every 0.5°. The primer pairs are listed in Table 2.

Bone microarchitecture analysis

BMD and the trabecular bone architecture were analyzed using micro computed tomography (micro CT; Guangzhou Zhongke Kaisheng Medical Technology Co., Guangzhou, China) with an isotropic voxel size of 1 m. The trabecular microarchitecture was scanned immediately in the proximal tibia. The trabecular bone was analyzed in 100 slices (1.0 mm total) to produce the data for analysis. Bone morphometric parameters were then quantified using the ZKKS-MCT-SHARP software. The bone parameters examined included the BMD, and the trabecular bone parameters included the trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), and structural model index (SMI).

Table 2. Primer pairs used for real-time RT–PCR

	Primers	
Gene	**Forward**	**Reverse**
Gapdh	5′-CCTGCACCACCAACTGCTTAGC-3′	5′-GCCAGTGAGCTTCCCGTTCAGC-3′
Runx2	5′-CTTCGTCAGCGTCCTATCAGTTC-3′	5′-TTCCATCAGCGTCAACACCAT-3′
Rankl	5′-TGGGCCAAGATCTCTAACATGA-3′	5′-TGGGATTTTGATGCTGGTTTT-3′
Nfatc1	5′-ACCAGCATTTCACGTACCTTCCT-3′	5′-GAGGCGCGGTGAGTTGTT-3′

Table 3. Animal weights (g)

Variable	Control (Mean ± SD)	HLS (Mean ± SD)	X-rays (Mean ± SD)	X-rays + HLS (Mean ± SD)
Weight[a]	200.08 ± 20.91	184.02 ± 19.58	203.68 ± 12.33	208.18 ± 19.01
Weight[b]	268.50 ± 22.09	239.52 ± 15.32	272.70 ± 19.96	194.32 ± 19.63**

Weight[a] = weight before the experiment, weight[b] = weight before sacrifice. *$P < 0.05$ compared with the control, **$P < 0.01$ compared with the control.

Histology

The femurs from the rats were stored in 10% formalin for 24 h and transferred to an EDTA solution for four weeks to extract the calcium [19]. They were cut into 4 μm sections at the General Hospital of Lanzhou Military Area Command to observe the morphology and quantities of trabecular bone with hematoxylin–eosin (HE) staining.

Statistical analysis

The experimental results were analyzed statistically with SPSS for Windows (version 20.0; SPSS Inc., IL, USA). Comparisons were made using one-way analysis of variance (ANOVA) and a paired-sample *t* test. The additive effects of HLS and radiation were tested with a factorial design. Unless otherwise stated, no statistical interaction was present. The Bonferroni test or Tamhane's test was performed to reveal significant differences between specific groups. Statistical significance was set at $P < 0.05$ for all comparisons. Data are shown as means ± standard deviation (SD), unless otherwise indicated.

RESULTS

Effects on animal mass

The mean animal mass at sacrifice for each group is shown in Table 3. There were no significant differences among the experimental groups in their initial animal masses (Table 3). However, a significant difference in animal mass was observed after treatment between the sham control and the X-rays + HLS groups.

Effects of treatments on trabecular bone

Compared with the sham control, the HLS group experienced significant deterioration of the trabecular microarchitecture. In the left tibia, the HLS group showed reduced BMD (–56.6%), Tb.N (–54.7%), and BV/TV (–65.7%), but increased Tb.Sp (+279.6%) and SMI (+170.2%). Only Tb.Th remained unchanged. The X-ray group showed significantly reduced BMD (–30.7%), Tb.N (–25.6%), BV/TV (–36.2%), and Tb.Th (–34.2%), but increased SMI (+105.3%). Tb.Sp remained unchanged. The HLS + X-ray group had the most affected trabecular parameters: significant reductions in BMD (–64.8%), Tb.N (–84.1%), BV/TV (–77.6%), and Tb.Th (–30.1%), but significant increases in Tb.Sp (+576.1%) and SMI (+223.7%). The synergistic effects of X-radiation and HLS on bone loss were evident as significant changes in BMD, Tb.Sp, Bv/Tv, and SMI when a factorial design was used (Fig. 1).

HE staining indicated the same trend, HLS was associated with worse trabecular parameters than the control group, the X-rays group showed a lesser affect when compared with the control group, while the HLS + X-ray group had the most affected trabecular (Fig. 2).

Induced expression of bone regulatory genes

We examined the control of osteoblast formation and the activity of osteoclasts in the production of regulatory factors, such as the receptor activator of NF-kB ligand (RANKL) and osteoprotegerin. The formation, maturation and activity of osteoclasts are stimulated by the RANKL/RANK receptor interaction and are downregulated by several factors [20–22]. Runx2 is a gene specifically expressed in osteoblasts. NFATc1 is a protein that regulates the proliferation and differentiation of osteoclasts. Once activated, it is translocated

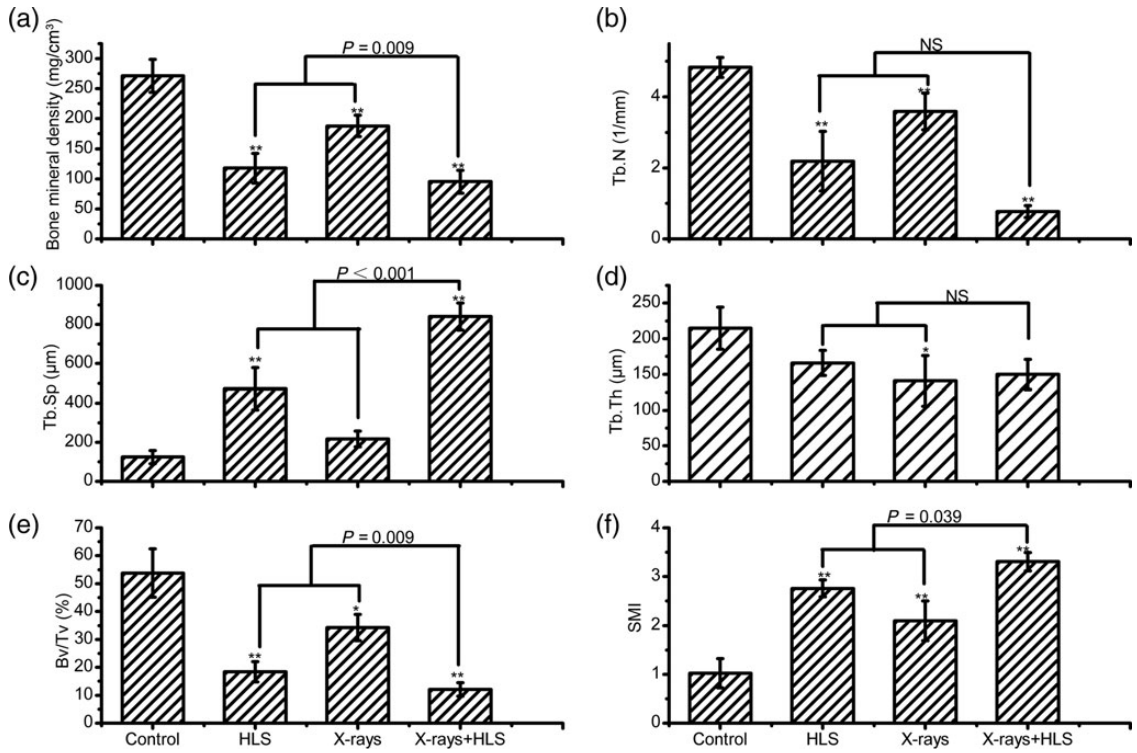

Fig. 1. Bone markers of the model groups. Trabecular parameters of the model groups in the order: control, HLS, X-rays, X-rays + HLS. The parameters include (**a**) bone mineral density, (**b**) trabecular number (Tb.N), (**c**) trabecular separation (Tb.Sp), (**d**) trabecular thickness (Tb.Th), (**e**) bone volume fraction (BV/TV), (**f**) structure model index (SMI). The arrow indicates the localization of gene expression. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control group.

into the nucleus and acts as a transcription factor for several osteoclast-specific genes [23].

In this study, the expression of Runx2 decreased significantly in the X-rays + HLS group (–64.9%). The expression of RANKL was more complex and increased in the HLS, X-rays and X-rays + HLS groups by 161.4, 46.7 and 20.1%, respectively. NFATC1 displayed the largest changes in the stressed animals, increasing in the HLS, X-rays and X-rays + HLS groups by +112.4, +156.9 and +147.9%, respectively. The X-rays + HLS group procedures clearly increased the expression of NFATC1 and RANKL compared with the control levels, whereas the reductions in RUNX2 expression were less significant (Fig. 3).

DISCUSSION

This study showed that after 4 Gy X-radiation and/or four weeks HLS, bone loss increased and the trabecular parameters changed markedly. The combined effects of HLS and X-rays were significant, and the X-rays + HLS group had the most affected parameters.

Changes in BMD caused by microgravity were observed very early in the history of space exploration, and recovery always requires a long time [24]. After the innovation of HLS by Dr Morey-Holton for the simulation of microgravity, studies of bone loss on the ground became possible, which clearly demonstrated that hindlimb suspension induces more bone loss than the disuse of limbs. Ionizing radiation has recently been identified as another major cause of spaceflight-induced bone loss with the dose rate of 0.6–1.2 Gy/min to simulate the space environment [25–26]. Several studies found that both hindlimb suspension and radiation can increase the numbers and resorptive surfaces of osteoclasts in cancellous tissues, whereas HLS and radiation did not exert combined effects [27]. However, in this study, we demonstrated the combined effects of HLS and ionizing radiation with a factorial design. Compared with the other experimental groups, the dual factors (X-rays + HLS) group showed the most deteriorative changes in their bones (Fig. 1). Because both HLS and ionizing radiation stimulate osteoclasts, they should display additive effects in enhancing bone loss.

The expression of RANKL and NFATC1 was significantly increased by the space-simulating environment; some researchers have shown that acute bone loss is predominantly caused by increased bone resorption rather than by reduced bone formation [28].

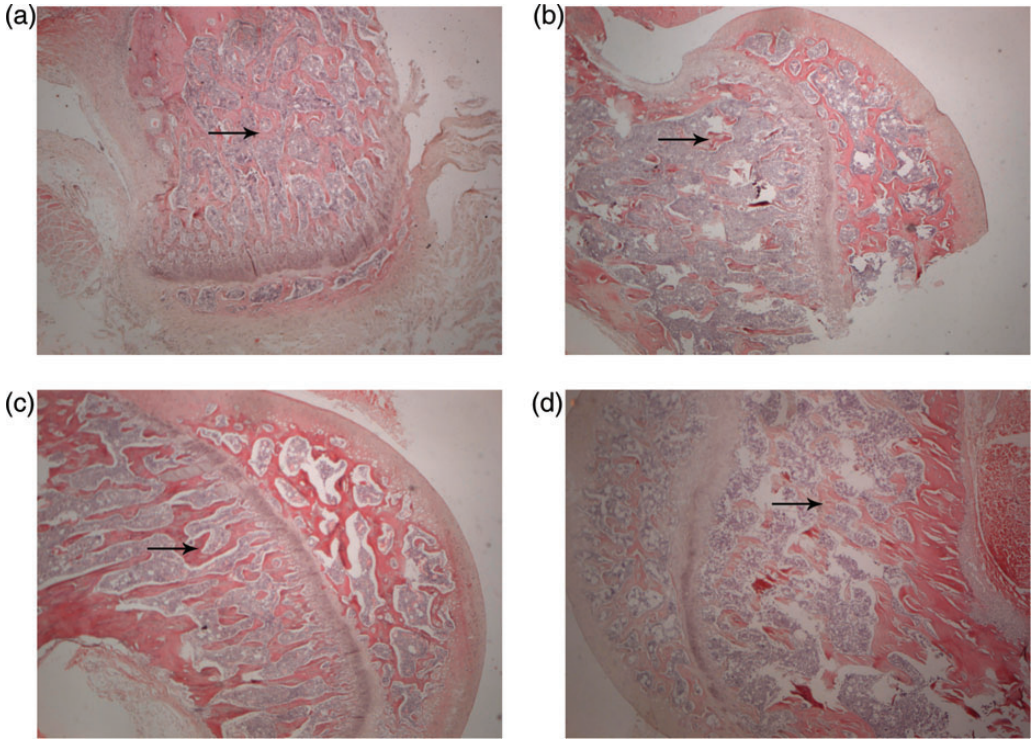

Fig. 2. HE staining of the model groups (×40) in the order: (**a**) control, (**b**) HLS, (**c**) X-rays and (**d**) X-rays + HLS; the arrow indicates the trabecular bone.

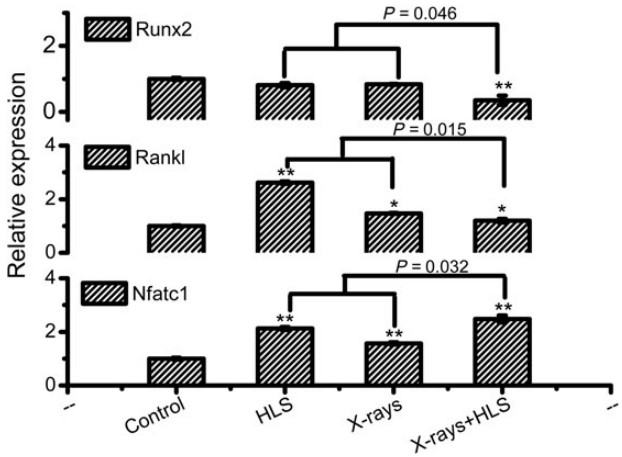

Fig. 3. Expression of RUNX2, RANKL and NFATC1. $*P < 0.05$ and $**P < 0.01$ compared with the control group.

Statistical analysis revealed that some of the effects of radiation were largely independent of HLS, suggesting that astronauts on long duration missions beyond the protective radiation shielding of Low Earth Orbit may be at greater risk of skeletal deterioration. Further study is needed to find a model in order to better understand the risks to skeletal health and aid in the development of effective countermeasures.

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